Sequence optimized real-time RT-PCR assay for detection of Crimean-Congo hemorrhagic fever virus

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Abstract

Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne virus of the genus *Nairovirus* within the family *Bunyaviridae*. Infection can result in general myalgia, fever, and headache with some patients developing hemorrhagic fever with mortality rates ranging from 5-30%. CCHFV has a wide geographic range that includes Africa, Asia, the Middle East, and Europe with nucleotide sequence variation approaching 20% across the three negative-sense RNA genome segments. While phylogenetic clustering generally aligns with geographic origin of individual isolates, distribution can be wide due to tick/CCHFV dispersion via migrating birds. This sequence diversity negatively impacts existing molecular diagnostic assays, leading to false negative diagnostic results. Here, we updated our previously developed CCHFV real-time RT-PCR assay to include CCHFV strains not detected using that original assay. Deep sequencing of eight different CCHFV strains, including three that were not detectable using the original assay, identified sequence variants within this assay target region. New primers and probe based on the sequencing results and newly deposited sequences in GenBank greatly improved assay sensitivity and inclusivity. Subsequent comparison of this assay to another commonly used CCHFV real-time RT-PCR assay targeting a different region of the viral genome showed improved detection, and both assays could be used to mitigate CCHFV diversity with diagnostics. Overall, this work demonstrated the importance of viral sequencing efforts for robust diagnostic assay development with specific improvement in our currently fielded CCHFV assay.

Introduction

Crimean-Congo hemorrhagic fever virus (CCHFV; family *Bunyaviridae*, genus *Nairovirus*) infection of humans can result in a disease spectrum ranging from a nonspecific febrile illness to hemorrhagic fever manifestations with a mortality rate of 5-30% [1]. The relatively low rate of disease in seropositive populations has spurred research into potential host susceptibility factors [2-5], although the availability of appropriate supportive care may provide a more direct correlation with clinical outcomes. CCHFV is predominantly transmitted by ixodid ticks of the genus *Hyalomma*, and CCHFV has a wide geographic distribution with endemic foci in eastern Europe, sub-Saharan and southern Africa, the Middle East, and Asia [6, 7]. Handling of tick-infested livestock and proximity to vegetated areas with high tick burdens are significant risk factors for CCHFV infection. In addition, nosocomial exposure to CCHFV-infected individuals in low resource facilities can result in severe disease among healthcare workers [1, 7].

CCHFV is an enveloped virus with a trisegmented, negative-sense RNA genome that encodes an RNA-dependent RNA polymerase (L), two major structural glycoproteins (G_N and G_C), and a nucleoprotein (N) on the L, M, and S genome segments, respectively. CCHFV has the largest genome of any bunyavirus at 19.1 kb total with 12.1, 5.4, and 1.6 kb in the three genome segments, respectively. To date, the NIAID Virus Pathogen Database and Analysis Resource (ViPR) lists complete genome sequences available for 54 (L), 75 (M), and 102 (S) genome segments of CCHFV isolates [8]. Pairwise alignments of these sequences indicate that their mean sequence identities are 89.4 % (L), 80.0 % (M), and 88.1 % (S).

Currently, there are no CCHFV vaccines or therapeutics approved for human use by the United States Food and Drug Administration, although immunoglobulin therapy and ribavirin have been used abroad with mixed results [14]. In the absence of approved countermeasures, effective diagnostics remain an invaluable means to identify and control CCHFV outbreaks. A variety of assay platforms for CCHFV can detect viral nucleic acids to include low density macroarrays [15], high density resequencing arrays [16], padlock probes with colorimetric readout [17], LAMP [18], and polymerase chain reaction [19-23]. Real-time reverse-transcription PCR remains the gold standard for quantitative, sensitive, and specific detection of CCHFV; however, these assays have sensitivity issues due to the genetic diversity of different CCHFV strains [24].

Previously, Garrison et al developed a TaqMan MGB real-time RT-PCR assay capable of detecting eighteen strains of CCHFV [25]. In subsequent testing of this assay identified several additional strains which were undetectable by this assay [20]. We suspected the inherent diversity of CCHFV genome contributed to inefficient primer/probe hybridization. To improve the assay performance, we sequenced these strains and designed a set of degenerate primers and probes to take into account CCHFV diversity in the assay target region. This optimization increased assay sensitivity compared to the original Garrison et al. assay and to a commonly used assay developed by Atkinson et al [20, 26-31].

Materials and Methods

Viruses. Multiple CCHFV strains including IbAr10200 (UCC# R4401), DAK8194 (UCC# R4416), SPU 128/81 (UCC# R4417), SPU 115/87 (UCC# R4448), UG 3010 (UCC# R4432), JD-206 (UCC# R4413), HY-13 (UCC# R4459), and Drosdov (UCC# R4405) were acquired from the Unified Culture Collection (UCC) maintained at US Army Medical Research Institute of Infectious Diseases (USAMRIID). Total RNA was extracted from 200 µl of cell culture supernatant using TRIzol LS (Thermo Fisher Scientific, Waltham, MA), the EZ1 Advanced XL (Qiagen, Valencia, CA), and the EZ1 Virus Mini Kit V 2.0 (Qiagen) according the manufacturers' recommendations. Total nucleic acid was eluted in 90 µl elution buffer and stored at -80 °C until use. Previously extracted RNA from additional CCHFV strains maintained at the USAMRIID including I-40, 2219 KKK28, I-248, SPU 97/85, SPU 134/ 87, SPU 415/85, and SPU 41/84 were used for assay inclusivity testing.

S segment sequencing and analysis. The S segment of each CCHFV isolate was amplified from 15 µl of total nucleic acid eluate using previously described primers [32] that were modified for Nextera-based Illumina sequencing. The sequencing and assembly of these segments were previously described (reference Genome Announcement). Briefly, the S segment of each virus was amplified using the SuperScript III One-Step RT-PCR system with Platinum *Taq* DNA Polymerase High Fidelity (Thermo Fisher Scientific) and gel-purified [QIAquick Gel Extraction Kit (Qiagen)]. Next-generation sequencing libraries were generated using the Nextera XT DNA Library Kit (Illumina) according to the manufacturer's instructions. Libraries were pooled and sequenced on the MiSeq Desktop Sequencer (Illumina). Consensus sequences were generated using CLC Genomics Workbench (Qiagen). The SRA files were deposited into Bioproject PRJNA360092, and the consensus sequences were deposited into GenBank [IbAr10200 (KY484036), DAK8194 (KY484027), SPU 128/81 (KY484044), SPU 115/87 (KY484040), UG3010 (KY484048), JD-206 (KY484037), HY-13 (KY484031), and Drosdov (KY484028)].

The S segments from these newly sequenced viruses were aligned, and the assay target region was isolated for variant analysis and assay redesign. Additionally, existing CCHFV S segment sequences from GenBank that covered the assay target region were aligned with CLC Genomics Workbench (Supplementary Figure 1).

Real-time RT-PCR assays. The existing CCHFV-S assay [25] was run as previously described with modifications described below. For the new assay described here (CCHFV-S2), primers and probe were designed within the same assay target region based on the data from the newly sequenced CCHFV isolates (see Table 1 for the primer and probe sequences and concentrations). Both assays (CCHFV-S and CCHFV-S2) were run on a Roche LightCycler 480 (Roche Applied Science, Indianapolis, IN) using the SuperScript One-Step RT-PCR Kit (Thermo Fisher Scientific), 5 µl purified nucleic acid, and a final concentration of 3 mM $MgSO_4$. Cycling conditions were 50 °C for 15 minutes, 95 °C for 5 min, and then 45 cycles of 94 °C for 1 s, 55 °C for 20 s, and 68 °C for 5 s. For the comparison with the Atkinson assay, primers, probe, and reaction conditions were the same as previously published [20]. The fluorescence was measured at the end of each 68 °C extension step, and a positive call required a quantification cycle (Cq) value of less than 40 cycles. All negative calls were given a Cq value of 40. The modified assay (CCHF-S2) was optimized for primer and probe concentrations using CCHFV IbAr10200 RNA. This process involved testing multiple primer concentrations ranging from 0.5 to 1.0 mM with 0.2 mM probe. The optimal primer concentration was selected based on the lowest Cq value and the highest endpoint fluorescence (data not shown).

A preliminary limit of detection (LOD) determination was conducted for both assays by serially diluting viral RNA either ten-fold or five-fold in two different series, and samples were run by real-time RT-PCR in triplicate. The preliminary LOD was the lowest RNA dilution where all replicates were positive. The LOD was confirmed by running 60 replicates at the LOD, requiring at least 58 of 60 replicates to be positive. Exclusivity testing was conducted using a viral RNA reference panel maintained at USAMRIID and acquired from the UCC. These viruses included Rift Valley fever virus (MP12), Hantaan virus (76118), yellow fever virus (17D), dengue virus serotype 1 (WestPac, UCC# R4423), dengue virus serotype 2 (S16803, UCC# R4424), dengue virus serotype 3 (CH53489, UCC# R4425), dengue virus serotype 4 (341750, UCC# R4426), West Nile virus [EG101 (UCC# R4310T) and NY99 (UCC# R4272T)], Chikungunya virus [B8636 and 38635), Lassa fever virus Josiah (UCC# R4086T), and Ebola virus variant Mayinga (UCC# R3828S). Inclusivity for both assays was determined using the 15 different strains of CCHFV maintained at USAMRIID and the UCC described above.

Statistics. Statistical analyses were performed using GraphPrism 6 (GraphPad Software, San Diego, CA). Assay linearity based on the preliminary LOD was determined based on the linear range of the curve using a nonlinear regression analysis. A two-way ANOVA with Sidak's multiple comparisons test was done to determine differences between the CCHF-S and CCHF-S-pan assay using multiple CCHFV strain RNAs.

Results

CCHFV strain sequencing and analysis. Since the development of our original CCHFV-S assay [25], we (CCHF-S, Table 2) and others [20] identified decreased assay performance including nondetection of several CCHFV strains (JD-206, Drosdov, and DAK8194). To address this problem, we conducted deep sequencing of multiple CCHFV S segments, including the three nondetectable CCHFV strains (reference Genome Announcement). The S segment consensus sequences for these viruses were aligned to identify mismatches within the assay target region (Figure 1).

Multiple nucleotide variants were identified in the primer and probe region for each strain sequenced (Figure 1), resulting in suboptimal primer/probe binding. Of note, a deletion in the 5' end of the published probe sequence, along with additional 3' probe variants for JD-206 and DAK 8194, likely resulted in nondetection of those two virus strains. Multiple variants in the reverse primer of Drosdov likely contributed to that strain's nondetection.

Assay evaluation. New primers and probe (assay CCHF-S2, Table 1) were redesigned to incorporate as much sequence diversity at the assay target location as possible. Comparison of the CCHFV-S2 assay primer/probe sequence to all CCHFV S segment sequences available in GenBank at the time of the assay redesign (n = 138, Supplementary Figure 1) showed the forward, reverse, and probe sequences had no greater than 1 mismatch (and an exact match within the last 2 bases of the 3' end) for 93.5, 99.3, and 98.6% of these S segment sequences, respectively.

The analytical characteristics of both the CCHF-S and CCHF-S2 assays were determined using a well-characterized stock of IbAr10200 (Figure 2, Table 3). The preliminary limit of detection (LOD), the highest dilution of virus where 3 of 3 replicates were all positive, was 1.28 PFU/reaction or 256 PFU/ml for the CCHF-S2 assay (Figure 2). Considering the linear segment of the dilution series, the R^2 value was 0.980, and the y-intercept was 43.64. This LOD was confirmed by running 60 replicates at this LOD, resulting in 58 of 60 positive replicates (Figure 2). For the CCHF-S assay using the same IbAr10200 RNA (Figure 2) identified the preliminary LOD, confirmed by 59 of 60 replicates being positive, to be 1.28×10^4 PFU/rxn or 2.56×10^6 PFU/ml. The assay linearity over the linear part of the dilution series was 0.845, and the y-intercept was 53.41.

The CCHF-S2 assay was then compared to another commonly used assay, the Atkinson assay [20], which targets a different region within the CCHFV S segment. Using the same RNA as a template and the reaction conditions described in [20], we identified a greater assay sensitivity compared to the CCHFV-S assay but decreased sensitivity compared to the CCHF-S2 assay (Figure 2, Tables 2 and 3). The assay LOD with IbAr10200 RNA was 2.56×10^4 PFU/ml with 60/60 replicates being positive. While several nucleotide variants were identified for the Atkinson assay within the assay target region of the CCHFV isolates previously sequenced (reference Genome Announcement), incorporating sequence-optimized reverse primer and the probe into the Atkinson assay did not change assay sensitivity (data not shown).

All three assays were then screened against an inclusivity panel of 15 different CCHFV isolates (Table 3). The CCHF-S2 assay and the Atkinson assay detected all of the CCHFV isolates including the three that the CCHF-S assay did not detect. Assay sensitivity, reflected in the Cq values, was generally better for the

CCHF-S2 assay (Table 3). In comparing the CCHF-S and the CCHF-S2 assays, almost all of these viruses had large improvements in the Cq values. For example, SPU 115/87 had ~10 Cq (~3 log) improvement in sensitivity, and IbAr10200 had ~15 Cq (>4 log) improvement (Table 3). Exclusivity testing for multiple viruses (see Materials and Methods) with the CCHF-S2 assay resulted in negative detection for each virus tested.

Discussion

Due to the low fidelity of the viral RNA-dependent RNA-polymerase, RNA viruses generally rapidly evolve under selective pressure, resulting in significant phylogenetic heterogeneity. This diversity can be problematic for diagnostic assays and therapeutics, requiring assay modification as additional sequence information becomes available. Indeed, two recently published studies investigating the genomic diversity of the Ebola virus circulating in West Africa [33, 34] identified multiple nucleotide variants among several commonly used Ebola virus real-time RT-PCR assays and therapeutics in development. These studies suggest such variants could negatively impact efficacy of diagnostic assays and therapeutics. To mitigate the diagnostic risk of CCHFV diversity, we re-designed our currently fielded CCHFV assay by incorporating sequencing data from several CCHFV isolates that were previously undetectable with the original assay.

Deep sequencing of the CCHFV S segments of these and other CCHFV isolates identified multiple nucleotide variants within the CCHF-S assay target region. These variants likely led to the decreased assay performance we observed the CCHFV-S assay. Variant analysis within this assay region did not identify intra-viral nucleotide differences (data not shown), suggesting some signature stability within each isolate and supporting continued targeting of this genomic region as a diagnostic signature. Based on these sequencing data and the CCHFV genomic data deposited into GenBank since the original assay design, a new assay (CCHF-S2) incorporated degenerate primers and probe taking into account the assay target sequence diversity. These primers greatly improved CCHFV detection, reflected in lower Cq values and detection of the three isolates not detected by the CCHF-S assay.

For highly diverse viruses like CCHFV, it is advantageous to have several diagnostic assays that target different regions of the viral genome in order to further minimize the diagnostic risk of a false negative call due to primer/probe mismatches. We conducted a comparison with another commonly used CCHFV assay developed by Atkinson and colleagues that targets the 5’ untranslated region of the CCHFV S segment [20]. While both the CCHF-S2 assay and the Atkinson assay positively detected all of the CCHFV strains tested here, the CCHF-S2 assay had improved sensitivity for most of the tested strains. Since both of these assays target different regions of the CCHFV genome, both assays could be used for increased confidence in diagnostic and biosurveillance efforts in order to mitigate the risk of nondetection due to CCHFV’s diversity.

In summary, we redesigned a CCHFV real-time RT-PCR assay that was initially developed when limited sequence information was available and did not perform optimally with newly acquired CCHFV isolates. This new assay contains degenerate primers and probe that accounts for a significant amount of the diversity within CCHFV, resulting in dramatically improved isolate detection and assay sensitivity. These data increase the confidence in the new assay detecting true positives, and this approach can be used to improve assay sensitivity existing nucleotide-based assays.

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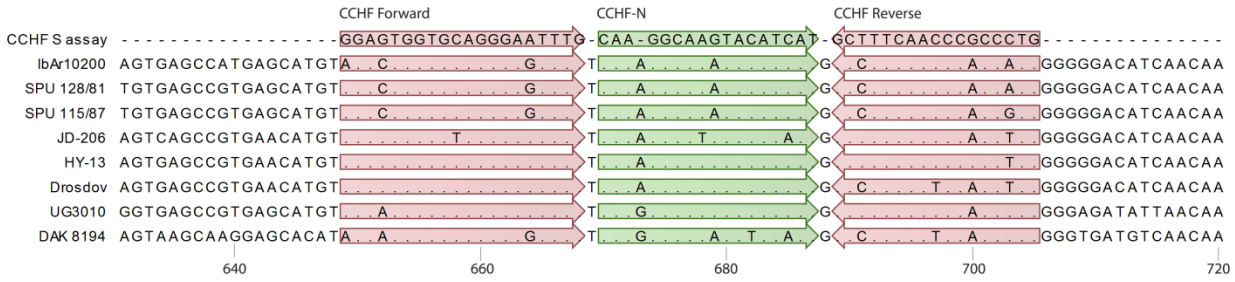

Figure 1. CCHFV isolate sequence analysis. Consensus sequences from eight newly sequenced CCHFV isolates (reference Genome Announcement) and the Garrison assay primer/probe sequences [25], indicated with red and green arrows, respectively, were aligned. Nucleotides identical to the primer and probe sequence are shown as dots, and nucleotide numbers are relative to IbAr10200. Degenerate primers and probe for the Garrison assay (see Table 1) were designed based off of these aligned sequences.

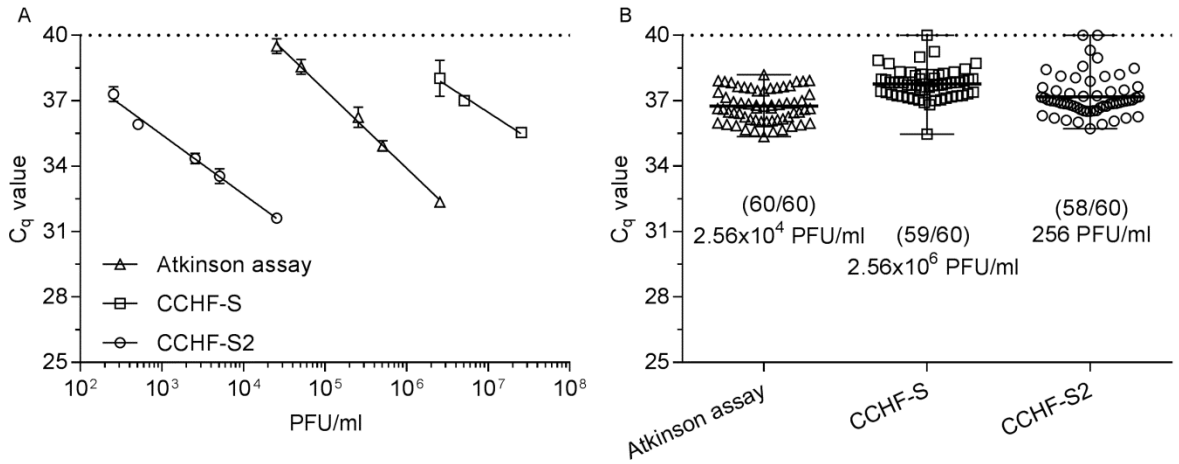

Figure 2. CCHFV assay characterization. (A) CCHFV IbAr10200 RNA was serially diluted in two series, 1:5 and 1:10, and assayed with the CCHFV assays. Shown is a nonlinear fit of the linear range where all three of the replicates were positive. (B) The preliminary LOD was confirmed by running 60 replicates at the preliminary LOD. The dashed line in each figure indicates the Cq positive/negative cutoff (40 cycles).

251
252
253

Table 1. CCHFV real-time primers and probe

Assay	Primers/pro	Sequence (5'-3')	Conc.	Amplicon	Referenc
CCHF-S	CCHF	GGAGTGGTGCAGGGAATTTG	1.25	57	[25]
	CCHF	CAGGGCGGGTTGAAAGC	1.25		
	CCHF-N	6FAM-CAAGGCAAGTACATCAT-	0.1		
CCHFV-	CCHF-SF2	GGAVTGGTGVAGGGARTTTG	1.0	57	here
	CCHF-SR2	CADGGTGGRTTGAARGC	1.0		
	CCHF-N2	6FAM-CAARGGCAARTACATMAT-	0.2		

Table 2. CCHFV assay detection

virus	detection (average Cq ± STDEV)		
	CCHFV-S2	CCHF-S[2]	Atkinson assay
I-40	20.66 ± 0.380	17.13 ± 0.242	27.62 ± 0.3
2219 KKK28	25.12 ± 0.04	22.34 ± 0.290	35.01 ± 0.16
I-248	22.95 ± 0.04	23.56 ± 0.174	31.23 ± 0.27
JD-206	34.29 ± 0.511	nd	31.69 ± 0.3
Drosdov	29.00 ± 0.091	nd	39.37 ± 0.64
HY13	19.66 ± 0.07	17.85 ± 0.11	24.94 ± 0.21
SPU 97/85	21.69 ± 0.133	34.10 ± 1.308	38.94 ± 0.51
SPU 134/87	25.48 ± 0.182	30.30 ± 2.081	31.61 ± 0.13
SPU 115/87	24.28 ± 0.489	34.35 ± 0.474	31.96 ± 0.26
SPU 415/85	18.97 ± 0.025	32.18 ± 0.219	26.81 ± 0.22
SPU 41//84	25.68 ± 0.083	32.86 ± 0.321	25.68 ± 0.2
SPU 128/81	23.16 ± 0.216	37.37 ± 0.525	28.97 ± 0.04
UG3010	24.20 ± 0.059	24.01 ± 0.050	29.84 ± 0.14
IbAr10200	24.28 ± 0.201	39.47 ± 0.924	30.55 ± 0.52
DAK8194	28.89/29.28[1]	nd	33.46 ± 0.54

[1]2 of 3 replicates were positive
[2]nd is not detected

Table 3. Analytical assay characteristics with IbAr10200

assay	linearity (R^2)	slope	y-intercept	LOD, PFU/ml (positives/60 replicates)	Cq ± STDEV	coefficient of variance
CCHF-S	0.845	-2.419	53.41	2.56×10^6 (59/60)	37.77 ± 0.68	1.79%
CCHFV-S2	0.980	-2.730	43.64	256 (58/60)	37.18 ± 0.91	2.95%
Atkinson assay	0.987	-3.581	55.4	2.56×10^4 (60/60)	36.75 ± 0.74	2.02%

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Supplementary

Figure S1. CCHFV sequence analysis. CCHFV S segment sequences containing the assay target region were identified in GenBank and aligned to show the forward and reverse primers (red) and the probe (green) region of the CCHF-S assay. The dominant nucleotide variants within these sequences, with the exception of one, are covered in the newly designed primers and probe. The single variant, the G at the 5' forward primer end, should have little impact on primer binding and extension.

CCHFV 10200 Standard Curve with Hewson assay

Run 10200 RNA in triplicate at 8 dilutions sarting at 1:10
with one NTC (H2O)

Reaction Mix
(SuperScript III Platinum One-Step Quantitative RT-PCR System)

				# Rxns =
Reagents	Stock	[Final]	1rxn	28
2X Reaction Mix	2	1	10	280
Nuclease-free water			1.7	47.6
100 µM F. Primer	18	0.9	1	28
100 µM R. Primer	18	0.9	1	28
100 µM Probe	25	0.625	0.5	14
RT/Platinum Taq Mix			0.8	22.4
X pg/µL RNA-pos cntrl/H2O		X pg/µL	5	
			20	

Cycling Conditions:

Temp	Time	Cycles
50C	10 min	1
95C	2 Min	1
95 C	10 sec	45
60 C	40 sec	
40 C	30 sec	1

Results:

Hewson

pfu/PCR rxn	CP			
Standard Log Concentration	Rep1	Rep2	Rep3	Avg
1.28E+04	32.25	32.21	32.61	32.36
2.56E+03	34.83	34.75	35.2	34.93
1.28E+03	36.33	36.66	35.73	36.24
2.56E+02	38.24	38.5	38.91	38.55
1.28E+02	39.47	39.17	39.86	39.50
2.56E+01	41.06	ND	40.88	40.97
1.28E+01	42.77	42.7	ND	42.74
2.56E+00	ND	ND	ND	

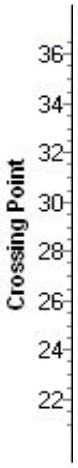
Error:	0.127
Efficiency:	1.994
Slope:	-3.337
Yintercept:	46.28

CCHFV Standard Curve

Run 10200 RNA in tripilcate at 7 dilutions starting with stock and one NTC (H2O)

Reaction Mix

				# Rxns =
Reagents	Stock	[Final]	1rxn	27
Mater Mix			14.6	394.2
RT/Platinum Taq Mix			0.4	10.8
X pg/µL RNA-pos cntrl/H2O		X pg/µL	5	
			20	



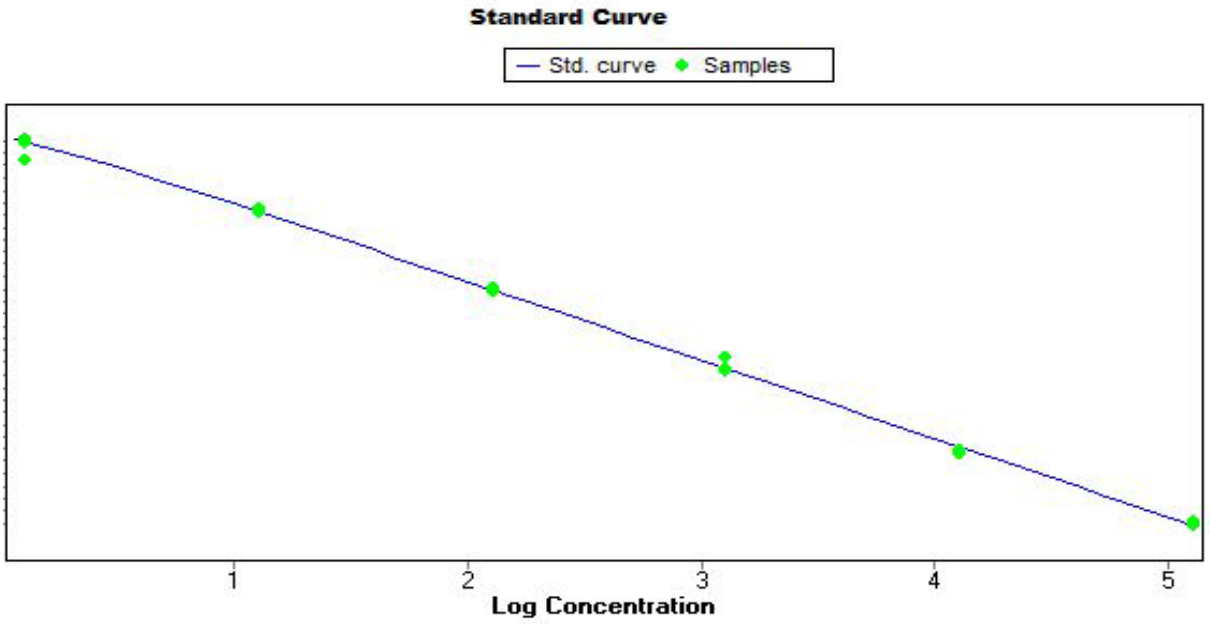
PCR Cycling Conditions

Temp	Time	Cycles
50C	15 min	1
95C	5 Min	1
94 C	1 sec	45
55 C	20 sec	
68 C	5 sec	
40 C	30 sec	1

Results:

	CP			
Standard Log Concentration	Rep1	Rep2	Rep3	
1.28E+05	21.04	20.87	21.03	20.98
1.28E+04	23.9	23.97	23.79	23.886667
1.28E+03	27.14	27.69	27.21	27.346667
1.28E+02	30.49	30.37	30.37	30.41
1.28E+01	33.55	33.77	33.71	33.676667
1.28E+00	36.44	36.57	35.74	36.25
1.28E-01	ND	ND	37.07	
1.28E-02	ND	ND	ND	

21.01	20.91	20.89	20.936667
24.1	23.99	24.2	24.096667
27.31	27.39	27.26	27.32
30.17	30.24	30.22	30.21
33.66	33.62	33.74	33.673333
35.74		35.84	35.79



x	y
1.28E+05	20.98
1.28E+04	23.88667
1.28E+03	27.34667
1.28E+02	30.41
1.28E+01	33.67667
1.28E+00	36.25

R^2

0.503227

X	Y
1.28E+05	21.04
1.28E+05	20.87
1.28E+05	21.03
1.28E+04	23.9
1.28E+04	23.97
1.28E+04	23.79
1.28E+03	27.14
1.28E+03	27.69
1.28E+03	27.21
1.28E+02	30.49
1.28E+02	30.37
1.28E+02	30.37
1.28E+01	33.55
1.28E+01	33.77
1.28E+01	33.71
1.28E+00	36.44
1.28E+00	36.57
1.28E+00	35.74

R^2

0.502586

Error:	0.023
Efficiency:	2.06
Slope:	-3.187
Yintercept:	36.95

1.28E+05	20.93667
1.28E+04	24.09667
1.28E+03	27.32
1.28E+02	30.21
1.28E+01	33.67333
1.28E+00	35.79

0.520809